Toxicology and Applied Pharmacology 302 (2016) 31–40

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

# Mitochondrial nucleoid clusters protect newly synthesized mtDNA during Doxorubicin- and Ethidium Bromide-induced mitochondrial stress

Lukáš Alán *, Tomáš Špaček, David Pajuelo Reguera, Martin Jabůrek, Petr Ježek

*Dept. 75, Institute of Physiology, Academy of Sciences of the Czech Republic v.v.i, Czech Republic*

ARTICLE INFO

*Article history:*
Received 20 January 2016
Revised 31 March 2016
Accepted 14 April 2016
Available online 19 April 2016

*Keywords:*
Doxorubicin
Ethidium Bromide
Nucleoid clusters
Mitochondrial DNA stress
Mitochondrial transcription factor A

ABSTRACT

Mitochondrial DNA (mtDNA) is compacted in ribonucleoprotein complexes called nucleoids, which can divide or move within the mitochondrial network. Mitochondrial nucleoids are able to aggregate into clusters upon reaction with intercalators such as the mtDNA depletion agent Ethidium Bromide (EB) or anticancer drug Doxorobicin (DXR). However, the exact mechanism of nucleoid clusters formation remains unknown. Resolving these processes may help to elucidate the mechanisms of DXR-induced cardiotoxicity. Therefore, we addressed the role of two key nucleoid proteins; mitochondrial transcription factor A (TFAM) and mitochondrial single-stranded binding protein (mtSSB); in the formation of mitochondrial nucleoid clusters during the action of intercalators. We found that both intercalators cause numerous aberrations due to perturbing their native status. By blocking mtDNA replication, both agents also prevented mtDNA association with TFAM, consequently causing nucleoid aggregation into large nucleoid clusters enriched with TFAM, co-existing with the normal nucleoid population. In the later stages of intercalation (>48 h), TFAM levels were reduced to 25%. In contrast, mtSSB was released from mtDNA and freely distributed within the mitochondrial network. Nucleoid clusters mostly contained nucleoids with newly replicated mtDNA, however the nucleoid population which was not in replication mode remained outside the clusters. Moreover, the nucleoid clusters were enriched with p53, an anti-oncogenic gatekeeper. We suggest that mitochondrial nucleoid clustering is a mechanism for protecting nucleoids with newly replicated DNA against intercalators mediating genotoxic stress. These results provide new insight into the common mitochondrial response to mtDNA stress and can be implied also on DXR-induced mitochondrial cytotoxicity.



## 1. Introduction

Mitochondria, the energy source and physiological regulators of eukaryotic cells, retain their own genetic information as a leftover of their α-proteobacterial ancestor. For example, human mitochondrial DNA (mtDNA) has 16.6 kbp and is compacted together with structural proteins into so-called nucleoids. The replication and transcription of mtDNA are managed by several proteins encoded by the nuclear genome. Indispensable replication proteins include DNA polymerase-γ (Polγ), Twinkle helicase, and mtRNA polymerase (Korhonen et al., 2004). Nucleoids contain at least two more essential proteins without catalytic activity, the mitochondrial (mt) transcription factor-A, TFAM, and mt single-stranded DNA-binding protein, mtSSB. The mtSSB protein forms tetramers and binds to single-stranded DNA during replication. Moreover, mtSSB also stimulates the enzymatic activity of both Polγ and Twinkle (Oliveira and Kaguni, 2010). The most efficient TFAM binding is exerted between the light strand promoter (LSP) and heavy strand promoter-1 (HSP1), where TFAM induces mtRNA transcription (Fisher et al., 1987; Ngo et al., 2011; Rubio-Cosials et al., 2011). Since mtDNA replication is primed by RNA, arising from transcription, TFAM also plays an important role in the initiation of mtDNA synthesis. TFAM binds to double-stranded DNA and bends its structure. Hence it, together with supercoiling mtDNA is compacted to a minimum space, while forming the framework of the typical nucleoid spheroid structure (Ngo et al., 2011; Rubio-Cosials et al., 2011). *In vitro*, TFAM binds to mtDNA by its two HMG boxes (Fisher et al., 1992) and imposes a U-turn on mtDNA (Ngo et al., 2011; Rubio-Cosials et al., 2011).

The replication of mtDNA and concomitant nucleoid segregation is a sensitive process, depending on perfect interplay between nucleoid replication factors. Several studies reported an impaired nucleoid segregation and the formation of nucleoid clusters induced by polγ overexpression (Di Re et al., 2009), Twinkle, and TFAM overexpression (Ylikallio et al., 2010), or TFAM (Kasashima et al., 2011) and Clpx protease downregulation (Kasashima et al., 2012). Changes in nucleoid distribution/segregation were also found during the metabolic switch

* Corresponding author at: Dept. 75, The Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14220 Prague 4, Czech Republic.
*E-mail address:* lukas.alan@fgu.cas.cz (L. Alán).

http://dx.doi.org/10.1016/j.taap.2016.04.011
0041-008X/

from glycolysis to oxidative phosphorylation in cybrid cell lines (Santra et al., 2004), and during the response to viral DNA (West et al., 2015).

Doxorubicin (DXR) is a drug used in cancer chemotherapy as an anthracycline antitumor antibiotic. DXR belongs to the anthracycline class and acts by intercalating DNA, with its most serious adverse effect being life-threatening heart damage. It is commonly used in the treatment of a wide range of cancers, including haematological malignancies (blood cancers, such as leukaemia and lymphoma), many types of carcinoma (solid tumours) and soft tissue sarcomas (Tacar et al., 2013). It is often used in combination chemotherapy as a component of various chemotherapy regimens.

Unfortunately, DXR has significant side effects on cardiomyocytes. Up to 48% of cancer patients receiving 700 mg/m$^2$ DXR developed cardiomyopathy (Swain et al., 2003). There are several hypotheses on the mechanism of DXR-induced cardiotoxicity. The first is based on DXR and its ability to produce reactive oxygen species (ROS) (Link et al., 1996). In the other hypothesis, DXR inhibits DNA topoisomerase IIβ (Top IIβ) and forms a covalent complex on DNA while forming double-strand breaks on mtDNA (Lyu et al., 2007).

Ashley and Poulton have shown that the pharmacological levels of DXR or Ethidium Bromide (EB), and other Top IIβ inhibitors and DNA intercalators also cause an aggregation of nucleoids of mitochondrial DNA (Ashley and Poulton, 2009). The cell response to DXR included remodelled nucleoids which eliminated DXR and maintained mtDNA synthesis, whereas non-remodelled nucleoids, still containing DXR, could not replicate. Fission and fusion cycles of the mitochondrial (mt) network were implicated in these responses (Ashley and Poulton, 2009). DXR and EB are DNA intercalators with different properties. Since DXR targets both the nuclear and mitochondrial genome, its cardiotoxicity can be linked to irreversible changes in mtDNA (Lebrecht et al., 2005). On the other hand, EB is a common DNA intercalator, binding preferentially to mtDNA. It is a well known agent in mtDNA depletion processes and in the creation of mtDNA-free (Rho zero) cells (King and Attardi, 1996).

Recently, the mechanism of TFAM cross-strand binding to mtDNA was revealed (Kukat et al., 2015) confirming the key role of TFAM in nucleoid packaging. Interestingly, a reduced amount of mtDNA by EB or TFAM knockdown in tumour cells increases their sensitivity to chemotherapeutic drugs such as DXR (Mei et al., 2015). These findings together with DXR-induced nucleoid clustering raise several questions, such as the role of TFAM in nucleoid clustering during mtDNA stress caused by DNA intercalators and what protect mtDNA against such stress.

Here, we hypothesized that TFAM is responsible for the clustering and consequently for the protection of replicated nucleoids. We used confocal microscopy in combination with biochemical studies to further characterize the mechanism of mitochondrial nucleoid aggregation due to the induction of drug-induced mtDNA stress which leads to nucleoid clustering accompanied by an initial depletion of TFAM binding to the stressed mtDNA. Recently replicated nucleoids are demonstrated to be preferentially accumulated in clusters, and thus protected by the accumulated TFAM and p53 protein. The nucleoid cluster formation is fully reversible; therefore we propose that such a stress response leads to the preservation of mtDNA genetic material.

## 2. Material and methods

### 2.1. Cell line and cultures

A human hepatocellular carcinoma HepG2 cell line (ECACC 85011430) was cultivated at 37 °C in a humidified atmosphere with 5% $CO_2$ in DME medium (DMEM, Life Technologies; contains no glucose) supplemented with 3 mM glutamine, 5 mM glucose, 10% (*v*/*v*) fetal calf serum (Biochrom AG, Berlin, Germany), 10 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The downregulation of selected genes (TFAM, mtSSB, mtRNA polymerase, ATG5, Lon protease, LonP or scrambled sequence) was carried out with purchased specific Silencer select siRNAs (Life Technologies) which were transfected using Lipofectamine RNAiMax (Life Technologies). The chemicals (Sigma-Aldrich, Saint Louis, MO) used for treatment of DNA were 500 ng/ml Ethidium Bromide, 3.4 μM Doxorubicin, 100 μM 2′,3′-dideoxycytidine, and 10 μM 5-bromo-2-deoxyuridine (BrdU). The rat cardiomyocytes were isolated at the department of developmental cardiology as described previously (Borchert et al., 2011).

### 2.2. Immunocytochemistry and Western blots

Cells fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min were then washed twice in PBS. All other steps were performed in PBS supplemented with 0.05% Triton X-100, 0.05% Tween 20 and 0.1 M glycine (all from Sigma-Aldrich), designed the washing buffer. Coverslips were incubated in the washing buffer with 5% donkey serum (Jackson ImmunoResearch, West Grove, PA) for 1 h. Subsequently, the coverslips were incubated with an appropriate antibody for 1 h, such as anti-TFAM (a kind gift from Prof. Bogenhagen, Department of Pharmacological Sciences, State University of New York at Stony Brook), anti-mtSSB (Sigma-Aldrich, HPA002866), anti-BrdU (Abcam, Cambridge, MA, AB8152; or Roche, Basel, Switzerland, 11296736001), anti-DNA (Progen Biotechnik, Heidelberg, Germany, 61014) or p53 (Sigma Aldrich, P6749). Samples were washed three times in the washing buffer and incubated with a secondary antibody, conjugated with Alexa Fluor 488 or Alexa Fluor 647, and 5% donkey serum for 1 h. Finally, samples were washed three times in PBS and used for confocal microscopy (Alán et al., 2010), or structured-illumination microscopy (SIM).

For Western blots, cells were lysed in the RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenyl-methyl-sulfonyl fluoride, and a protease inhibitor cocktail, Sigma Aldrich). Protein concentrations were determined using BCA Protein Assay Reagent (Pierce, Rockford, IL) before they were loaded onto SDS–polyacrylamide gels. Separated proteins on gels were transferred onto PVDF membranes, subsequently treated with the relevant primary antibody and then with secondary, horseradish peroxidise-conjugated antibodies. The detection was done using an Amersham ECL plus blotting kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The following antibodies were used: Tim23 (BD Biosciences, San Jose, CA, 611222), Mitofilin (Abcam, AB110329), TFAM (kindly provided by Prof. Bogenhagen), mtSSB (Sigma-Aldrich), Twinkle (Abcam, AB83329), DRP1 (BD Biosciences, 611112), ND5 (Sigma Aldrich, SAB2101558) and LC3B (Novus Biologicals, Littleton, CO, NB600-1384). A quantitative analysis of light intensity was performed by densitometry using the software ImageJ. The obtained protein levels were normalized to Coomassie blue staining (Gilda and Gomes, 2013) and are shown in Supplementary data Fig. 2.

### 2.3. Real time PCR

After appropriate treatment, HepG2 cells were collected by trypsinization and frozen until processed for DNA isolation; or cells were directly lysed with TRIZOL (Life Technologies) for RNA isolation. DNA samples were dissolved in 600 μl of the lysis buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.1% SDS). Lysates were treated with proteinase K (Sigma Aldrich) for 3 h and then mixed with phenol–chloroform–isoamyl alcohol (25:24:1, vol.,vol.,vol.) and spun down at 10,000 ×g for 10 min. The upper aqueous phase was collected, mixed with chloroform-isoamyl alcohol (24:1, vol.,vol.), and spun down again. The upper aqueous phase was collected and mixed with 10% of 3 M sodium acetate and one volume of isopropanol. Samples were mixed, stored overnight in the freezer and then centrifuged at 19,000 ×*g* for 20 min at 4 °C. The obtained pellets were washed in 70% ethanol then centrifuged again, air-dried and dissolved in the